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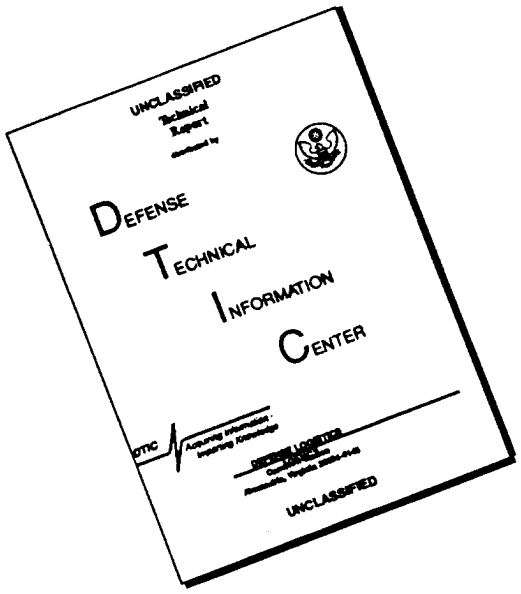

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Breast cancer is a disease where breast epithelial cells become refractory to appropriate growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulators element already implicated in some breast malignancies and on a class of negative regulators of cyclin-dependent kinases (Cdks). Preliminary data indicate that both cyclin E and Cdk inhibitors are important in breast malignancy.

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(5) INTRODUCTION

Breast cancer is a disease where breast epithelial cells become refractory to growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulatory element already implicated in some breast malignancies and a class of negative regulators of cyclin dependent kinases (Cdks). To study the role of cyclin E in breast malignancy, we screened cell lines derived from breast carcinomas for cyclin E mutations. We have also created cyclin E mutations and studied their effects on cultured cells. Additionally, we have targeted cyclin E using antisense strategies to examine its essentiality. To investigate the role of Cdk inhibitors in regulation of proliferation of breast epithelial cells, we have (1) identified and studied Cdk inhibitors in other cell types where analysis is more straightforward and (2) attempted to extrapolate these results to breast epithelial cells to determine the relevance for cell cycle control in this system.

(6) BODY

(a) SOW Task 1: Characterization of Cdk inhibitor production in normal and malignant breast epithelial cells (months 1-24).

We could not find a critical role for known Cip/Kip family members in normal breast epithelial cells responding to cell cycle regulatory signals as compared to cell lines derived from mammary tumors (data not shown). On the other hand,

others have found that regulation of a different type of Cdk inhibitor, p15, is likely to be important for cytokine regulation of epithelial cells in general (1) and breast epithelial cells, in particular (J. Slingerland, personal communication). Therefore we have considered this avenue as no longer sufficiently productive to merit further work and are focusing on other approaches.

Methods: The methods employed were standard preparation of protein lysates from mammalian tissue culture cells and Western blotting by the ECL method.

(b) SOW Task 2: Characterization of cyclin/Cdk complexes in normal and malignant breast epithelial cells (months 1-36).

We have characterized cyclin E/Cdk2 and cyclin A/Cdk2 complexes from normal breast epithelial cells and a number of cell lines derived from breast carcinomas for responsiveness to Cip/Kip family inhibitors. We found that one cell line, MDA-MB-231, produced a cyclin E/Cdk2 but not a cyclin A/Cdk2 that was reproducibly resistent to inhibition (see previous year's report). This led us to infer that the basis for the resistence was likely to be an alteration of cyclin E structure or function rather than of Cdk2 structure or function. We, therefore, cloned the cyclin E cDNA from MDA-MB-231. Several clones from this cell line contained a three-codon deletion of amino acids 23 through 25, suggesting that the mutant cyclin E encoded might account for resistance to the inhibitors.

We therefore expressed both the mutant and wild-type cyclin E in yeast cells containing human Cdk2, immunoprecipitated cyclin E/Cdk2 complexes and tested their sensitivity to inhition by recombinant p27 in the context of a histone H1 kinase

assay. The results of one such assay are shown in Fig. 1. Clearly, there is no significant difference between the recombinant mutant and wild-type cyclin E/Cdk complexes in terms of their sensitivity to p27. We must therefore conclude that differences in inhibitor sensitivity of endogenous cyclin E complexes prepared from MDA-MB-231 are due to other proteins in the lysates that remain to be characterized.

Methods: The PCR product obtained from MDA-MB-231 containing the mutant allele of cyclin E was cloned in yeast expression vector YCpG2 and transformed in parallel with the equivalent wild-type clone into a yeast strain where the endogenous Cdk, Cdc28, had been substituted with human Cdk2. Immunoprecipitations and cyclin E kinase assays were as described (2). Recombinant hexahistadine-tagged human p27 was prepared from E. coli, as described (3). Kinase activity was measured for mutant and wild-type cyclin E complexes with increasing amounts of p27.

(c) SOW Task 3: Test of essentiality of cell cycle regulatory components in breast epithelial cells by antisense (months 1-12).

We are employing three antisense methods to test for essentiality of cyclin E and, eventually, other proteins: antisense deoxyoligonucleotides, antisense ribozymes and antisense-encoding adenoviruses. Although initial results using oligonucleotides were discouraging, we have now obtained reasonable results using C-5 propyne-modified pyrimidine substituted oligonucleotides. Fig. 2 shows a cyclin E western blot corresponding to a titration of such a cyclin E antisense oligonucleotide using the human breast epithelial cell line 184A. As can be seen, the antisense oligonucleotide significantly reduces the level of cyclin E relative to

the control oligonucleotide. To assess the biological consequences of reducing cyclin E levels, the same transfected cell populations were analyzed for alterations in cell cycle distribution 24 hours after transfection (Fig. 3). Even though cyclin E levels were reduced to approximately 20% of control transfected cells, there was no significant apparent effect on the rate of entry into S phase. Therefore, it appears that cyclin E levels are not rate-limiting for entry into S phase in breast epithelial cells. We cannot, however, determine from these data whether cyclin E is essential in these cells, since antisense oligonucleotides are not sufficiently efficient to completely eliminate the protein. Hence, our data are not directly in conflict with antibody microinjection experiments that indicate that cyclin E is essential in fibroblasts (4).

Methods: An oligonucleotide (15-mer) was synthesized corresponding to nucleotide 543 to 557 of the cyclin E cDNA (Fig. 4) was transfected into asynchronous 184A cells using cationic lipid. Cells were either harvested to prepare protein for Western blotting or for FACS analysis at 24 hours post transfection.

In the hope of obtaining a more complete elimination of cyclin E, we are pursuing the antisense ribozyme approach. This has proven technically difficult but progress has been made. Using the random antisense library approach of Lieber and Strauss (5), a strong, ribozyme-accessible cleavage site was found in cyclin E mRNA. Although, the product of the cleavage mapped to a particular potential hammerhead ribozyme cleavage site in the cyclin E mRNA, we are constructing ribozymes corresonding to several potential cleavage sites in this region of the mRNA, reasoning that this entire region may be structurally accessible. The target sites to be used are indicated in Fig. 4.

In order to target cyclin E mRNA in breast epithelial cells, the tetracycline repressible system (6) was introduced into the 184A breast epithelial cell line. In order to insure stability of ribozymes once expressed, an expression vector was developed that embed the antisense ribozyme sequence in the VA1RNA sequence of adenovirus (5). This RNA is known for its *in vivo* stability. In order to adapt it ot the context of conditional expression using the tetracycline system, strong polIII promoter elements were deleted from the vector sequences. Ribozyme clones have been prepared and transfected into the recipient 184A cell line. Tranfectants will be analyzed for modulation of cyclin E and phenotype in the near future.

Methods: standard molecular biology procedures were employed.

In addition to ribozymes, we have recently begun utilizing antisense expressing recombinant defective adenoviruses as an alternative approach. This method has the advantage of achieving regulated high uniform levels of antisense expression in total populations of cells. Recombinant adenovirus stocks expressing the complete cyclin E cDNA in antisense orientation have been prepared and will be transduced into 184A breast epithelial cells to assess the effects on cyclin E levels and to determine phenotype in the near future.

Methods: Recombinant adenoviruses are prepared by co-transfecting a plasmid containing an expression cassette embedded in adenovirus sequences with a fragment containing most of the adenovirus genome into 293 cells, which can complement the missing viral functions. Recombinant viruses are placque purified, amplified and purified by cesium cholride banding.

Since several reports suggest that abnormal accumulation of cyclin E may have a role in the etiology of breast cancer (7,8), we have also taken the liberty of investigating the physiology of cyclin E accumulation. Even though this was not explicit in the original proposal, we feel that it is well within the scope of the project.

We identified cyclin E mutations that lead to hyperaccumulation of cyclin E, analysis of which indicated that cyclin E ubiquitination and turnover are regulated by autophosphorylation of cyclin E/Cdk2 on Thr380 of cyclin E (3). Mutating this residue leads to increased half-life and persistent accumulation of cyclin E in mammalian cells (Fig. 5). We have now shown that the consequences of this is perturbed progression through S phase and mitosis and, more importantly, genetic instability. Conditional expression of stabilized mutant alleles of cyclin E in Rat-1 fibroblasts, particularly a C-terminal truncation, allows enhanced gene amplification at the CAD locus (Table 1). We are now performing similar experiments in 184A human breast epithelial cells.

Methods: Standard molecular biological approaches were employed. Mutant and wild type cyclin E alleles were expressed in Rat-1 fibroblasts or 184A breast epithelial cells using the tetracyclin repressible system. Genetic instability was measured by plating cells in medium containing PALA, an inhibitor of aspartate transcarbamoylase, resistence to which measures amplifcation at the CAD locus.

(d) SOW Task 4: Cloning and characterization of Cdk inhibitors from HeLa cells (months 1-36).

As reported previously, we characterized a Cdk inhibitor activity from HeLa cells and demonstrated that it corresponded to $p27^{Kip1}$. Analysis of the regulation of p27 in HeLa cells and in normal human diploid fibroblasts indicated that p27 accumulation in response to drugs was mediated by translational control (9). p27 accumulation in response to contact inhibition was shown to be mediated both at the level of translational control as well as modulation of the half-life of the protein (9) (Fig. 6). In neither instance was transcriptional control implicated.

To explore the mechanism of translational control, we have investigated a potential role for pp70 S6 kinase. We have shown that in contact inhibited fibroblasts and lovastatin treated HeLa cells, conditions where p27 translation is inreased, S6 kinase is inhibited (Fig. 7). The role of S6 kinase in p27 translation is being tested using an *in vitro* translation system. We are also analyzing the long 5' untranslated region of the p27 mRNA for a possible role in translational control. The 5' UTR of p27 contains both a polypyrimidine tract (underlined in Fig. 8) and a long inverted repeat predicted to from a stem loop (opposing arrows in Fig. 8). These are being mutated and evaluted for translational regulation *in vivo*.

Methods: S6 kinase activity was measured by immunoprecipitating pp70 S6 kinase and testing for phosphorylation of the S6 subunit of purified 40S ribosomes. The complete 5'UTR of human p27 was cloned and sequenced by standard molecular biological methods.

(e) SOW Task 5: Cloning and characterization of breast epithelial cell Cdk inhibitors (months 12-48).

Since p27, cloned based on our work in HeLa cells, did not appear to be relevant to regulation of normal breast epithelial cells, we sought other potential inhibitors from breast epithelial cells. We noticed that an antibody prepared against a peptide corresponding to a conserved sequence in Cip/Kip family inhibitors reacted with a 25kD polypeptide in non-immortalized human breast epithelial cells. Two approaches were used to characterize this protein: attempts were made to clone a cDNA corresponding to it from human breast cDNA and it was tested directly for activity as a Cdk inhibitor.

We initially demonstrated that p25 was resistent to boiling, as are other members of the Cip/Kip inihibitor family. We then prepared boiled lysates from breast epithelial cells and performed SDS-PAGE gel elution experiments to test for Cdk inhibitory activity (Fig. 9). Based on these experiments, we concluded that p25 has no Cdk inhibitory activity. We now think that p25 is an inactive fragement of the Cdk inhbitor $p57^{Kip2}$ (see below). On the other hand, p21 present in these cells is clearly detectable by this assay (Fig. 9).

Methods: Boiled lysates were prepared, subjected to SDS-PAGE, eluted, renatured and tested for kinase inhibitory activity as described previously (2).

Using a cDNA library from human breast epithelial cells and degenerate primers predicted to amplify sequences corresponding to members of the Cip/Kip inhibitor family we were able to isolate fragements corresponding only to p21, p27 and p57 (data not shown). Upon reexamination of the expression pattern of p25 in human breast epithelial cells, we noticed that it closed paralleled the pattern of expression of p57. We further noticed that accumulation followed the approach of cells to senescence. Our interpretation of these observations, therefore, is that p25

is likely to be either a proteolysis or alternative splice product of p57 that has no Cdk inhibitory activity and the accumulation of p25 and p57 is a consequence of these cells becoming senescent in culture.

To circumvent the problems inherent in cDNA cloning and to identify new members of the Cip/Kip family that might have implications for breast epithelial cell biology, we have probed a human genomic phage library with a labelled degenerate oligonucleotide correponding to a conserved sequence of Cip/Kip family inhibitors. So far, three independent reactive genomic sequences have been identified that do not correspond to p21, p27 or p57. These are presently being sequenced.

Methods: Standard molecular biology methods were employed.

(f) SOW Task 6: Characterization of Cdk inhibitors in vivo (months 24-48). We have not yet initiated this phase of the project.

(7) CONCLUSIONS

We could provide no compelling evidence that our initial hypothesis that Cip/Kip family Cdk inhibitor proteins were important for regulation of the cell cycle in human breast epithelial cells. Although these cells express p21 and p27 to some degree, although they do not seem to be implicated directly in the regulatory modes that we investigated. p57, and a probable inactive derivative, p25, were also present in breast epithelial cells, but appear to be a symptom of approaching senescence. On the other hand, p15, a member of INK4 family of Cdk inhibitors appears to be involved in cytokine-mediated cell cycle arrest of breast epithelial cells. However,

since others are pursuing this aspect of inhibitor action, we have chosen not to. Instead we are probing genomic libraries in the hope of finding new Cdk inhibitors that might have a role in regulation of breast epithelial cells.

With regard to analysis of Cdk complexes from breast tumor derived cell lines, we found that cyclin E/Cdk2 complexes from one MDA-MB-231, were resistent to inhibition by members of the Cip/Kip family. We cloned a cyclin E variant from this cell line that had a three-amino acid deletion. However, when recombinant cyclin E complexes were prepared containing this variant or wild type, there was no difference between the two, indicating that resistence to inhibitors of the endogenous complexes must be due to other, as yet, indeterminate factors.

Since we cloned human p27 in the context of this project, we have continued to investigate its regulation. In particular, we have determined that translational control rather than transcriptional control is the primary mode of regulation in many different cell cycle regulatory contexts. Correlative data suggest that regulation of ribosomal function by pp70 S6 kinase may be involved, although the regulation of p27 translation by this kinase would be the converse to what has been demonstrated for other translationally-regulated mRNAs so far. In addition, analysis of the 5' UTR of the p27 mRNA is consistent with possible translational regulation.

A major effort is now being focused on understanding the function and regulation of cyclin E. Antisense work has suggested that cyclin E is not normally rate-limiting for the G1 to S phase transition in breast epithelial cell lines, which runs counter to accepted dogma for mammalian cells, in general. We are trying to perfect antisense ribozyme and antisense adenovirus approaches in order to

eliminate cyclin E expression completely and to thus determine if cyclin E is essential for the G1 to S phase transition in breast epithelial cells. This is an important issue if one is to consider targeting cyclin E/Cdk2 in a therapeutic context. A complementary issue that we are focusing on is whether abnormal accumulation of cyclin E might be important in the context of malignant transformation and breast cancer, in particular. We have found that point mutations in cyclin E can stabilize the protein dramatically in vivo and, more interestingly, lead to genetic instability. Proper regulation of cyclin E levels is of particular relevance to breast cancer since transgenic mice that overexpress cyclin E in the mammary epithelium during pregnancy and lactation develop a high incidence of mammary carcinoma (8). Therefore, we are investing a signicant effort in understanding how deregulation of cyclin E accumulation leads to loss of cell cycle control and genetic instability. We will also analyze cyclin E from tumor derived material to determine if stabilizing mutations are present, explaining in part the transformed phenotype.

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Table 1.

Cyclin E genotype[1]	Induction	PALA^r Frequency[2]	Fold Resistance
wild-type	-	4.6×10^{-5}	
	+	7.0×10^{-5}	1.5
T380A	-	2.6×10^{-5}	
	+	5.1x10-5	2.0
C-term truncation	-	1.6×10^{-4}	
	+	1.2×10^{-3}	7.5

[1] All three cell lines were high expressors of the respective cyclin E alleles.

[2] All cell lines were induced for cyclin E expression for 32 days followed by 21 days of selection in PALA at a concentration of LD_{50}x7. The frequency of PALA resistance was calculated as the PALA resistant colonies out of colony forming units plated (number cells plated x colony forming efficiency).

Figure Legends:

Figure 1. The three-codon deletion variant of cyclin E does not confer a change in sensitivity of cyclin E/Cdk2 histone H1 kinase to inhibitor p27. Equivalent immunoprecipitations containing either wild-type cyclin E or a nine nucleotide deleted variant (δ 9 cyc E) complexed to Cdk2 were treated with increasing amounts of recombinant p27 (arbitrary units). Values are percentages of control assay without p27 and are based on PhosphorImager scans of labelled histone H1 bands.

Figure 2. Treatment of human breast epithelial cells (184A) with a C-5 propyne substituted phosphorothioate cyclin E antisense oligonucleotide reduces the level of cyclin E protein. Asynchronous cells were transfected with two different concentrations of either antisense or control oligonucleotides. After 24 hours, cells were harvested and extracts separated by SDS-PAGE. Equivalent amounts of protein were loaded per lane and cyclin E was detected by Western blotting.

Figure 3. Reduction of cyclin E levels does not significantly affect the distribution of cells in the cell cycle . Human breast epithelial cells (184A) were transfected at a concentration of 1 nM or 3nM with either anti-cyclin E or control oligonucleotide. After 24 hours, cells were harvested, stained with propidium idodide, and analyzed by FACS. All populations were indistinguishable, indicating no significant change in rate of passage through the various cell cycle phases. Previous studies demonstrated no alterations in the rate of cell proliferation at these oliognucleotide concentrations.

Figure 4. Sites in the human cyclin E targeted by antisense oligonucleotide and ribozymes based on ribozyme library cleavage data. The boxed sequence corresponds to the oligonucleotide target and the underlined sequences correspond to the ribozyme targets. Bold letters correspond to the specific triplet targeted by each ribozyme. Numbering corresponds to nucleotides of the cyclin E cDNA.

Figure 5. Mutation of Thr380 of human cyclin E increases the its half-life in Rat-1 fibroblasts. Rat-1 clones expressing wild-type human cyclin E or a T380A mutant allele were pulse labelled with ^{35}S methionine, followed by a cold chase. Lysates were prepared at the indicated times and cyclin E was immunoprecipitated and analyzed for loss of label using a phosphorImager after SDS-PAGE. o, wild-type cyclin E; Δ, T380A.

Figure 6. Translational control of p27 in human diploid fibroblasts. (A) a pulse-chase experiment was performed with human diploid fibroblasts (HS68). Contact inhibited or asynchronous cells were pulse labelled for one hour with ^{35}S methionine and subsequently chased with an excess of cold methionine for the times indicated. Cells were lysed and p27 immunoprecipated followed by analysis by SDS-PAGE and autofluorography. (B) Asynchronously growing HS68 fibroblasts were incubated with ^{35}S methionine for 90 minutes followed by a cold chase and p27 was analyzed as described above. (C) Contact inhibited or asynchronously growing HS68 fibroblasts were pulse-labelled with ^{35}S methionine for 30 minutes, and p27 was analyzed as described above.

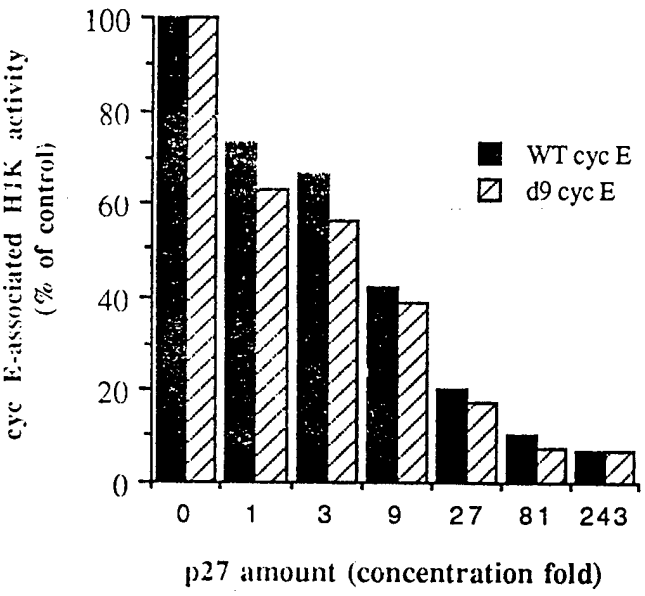
Figure 7. Lovastatin inhibits pp70 S6 kinase in HeLa cells. pp70 S6 kinase was immunoprecipitated from lyasates prepared from either asynchronous HeLa cells or HeLa cells that had been treated with lovastatin for 24 hours. Kinase assays were

performed by incubation with purified 40S ribosomal subunits and γ-^{32}P ATP followed by SDS PAGE and autoradiography. The arrow indicates the position of the substrate, S6.

Figure 8. The coding strand of the region of the human p27 cDNA corresponding to the 5' untranslated region (UTR) of the mRNA. The 5' pyrimidine tract is underlined. The palindrome predicted to form a hairpin is indicated by the inverted arrow. The initiation codon is in italics.

Figure 9. Gel elution analysis of breast epithelial cell lysates for Cdk inhibitor activities. Lysates from EGF starved breast epithelial cells (184) were boiled, precipitated proteins pelleted and supernatants separated by SDS-PAGE. Gels were sliced and proteins eluted and renatured as described (2). Cyclin E/Cdk2 immunoprecipitates were incubated with renatured proteins from each slice and then assayed for histone H1 kinase activity. Histogram indicates the quantitated activity (based on PhosphorImager scans) for each reaction. Control reactions are indicated at the right. Positions of molecular weight standards are also shown. Significant inhibition activity in these lysates is detectable only for p21. No inhibitory activity is detectable for p25.

Figure 1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p27 amount (concentration fold)

Figure 2

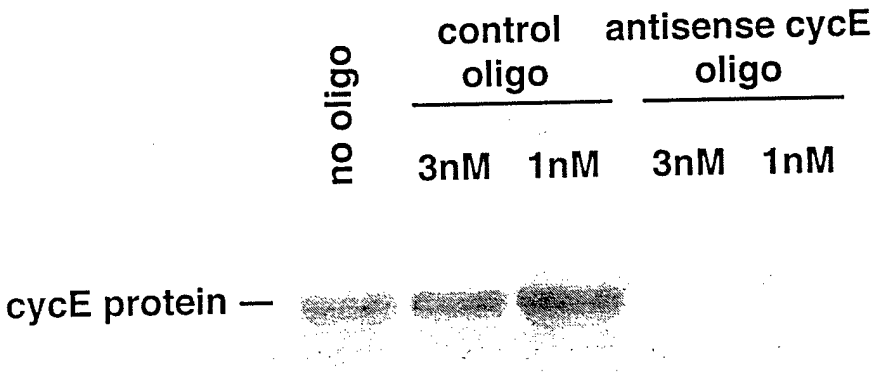
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Figure 3

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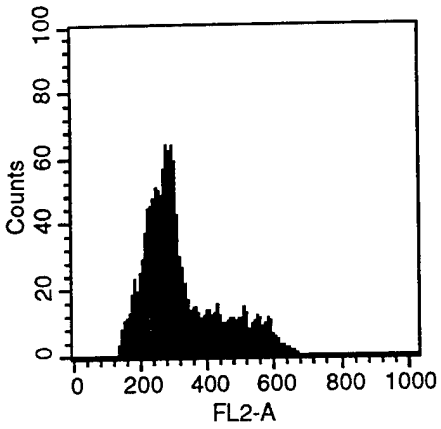

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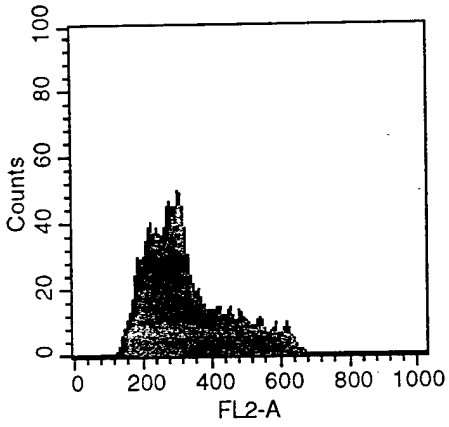

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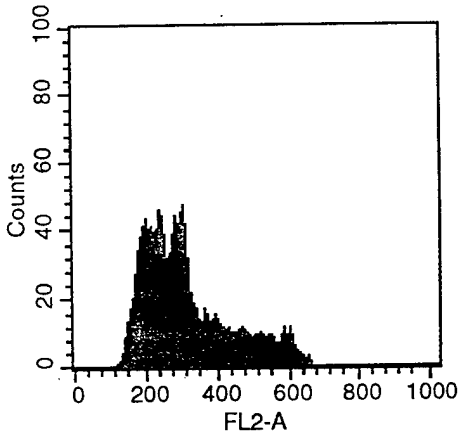

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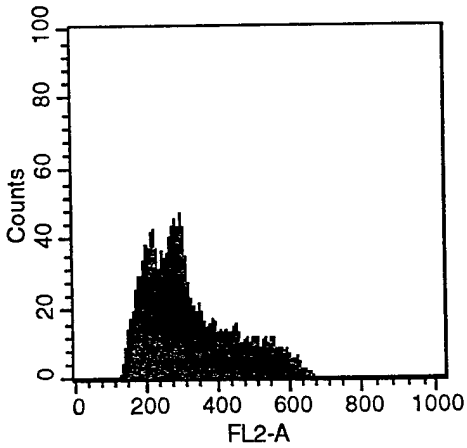

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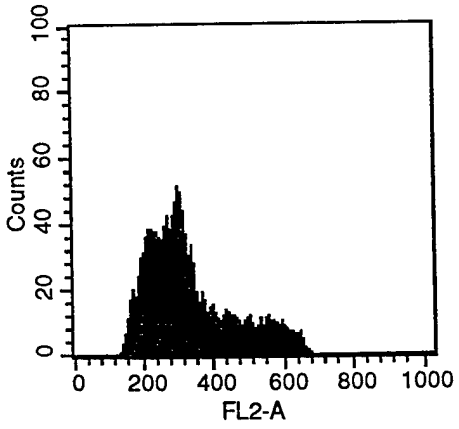

Figure 4

Position of Antisense Ribozymes and Oligonucleotide Targets

58 atgaaggagg acggcggcgc ggagttctcg gctcgctcca ggaagaggaa ggcaaacgtg

118 accgtttttt tgcaggatcc agatgaagaa atggccaaaa tcgacaggac ggcgagggac

178 cagtgtggga gccagccttg ggacaataat gcagtctgtg cagacccctg ctccctgatc

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718 gcc**ctt**aagt ggcgtttaag tcccctgact attgtgtcct ggctgaatgt atacatgcag

778 gttgcatatc taaatgactt acatgaagtg ctactgccgc agtatcccca gcaaatcttt

838 atacagattg cagagctgtt ggatctctgt gtcctggatg ttgactgcct tgaatttcct

898 tatggtatac ttgctgcttc ggccttgtat catttctcgt catctgaatt gatgcaaaag

958 gtttcagggt atcagtggtg cgacatagag aactgtgtca agtggatggt tccatttgcc

1018 atggttataa gggagacggg gagctcaaaa ctgaagcact tcaggggcgt cgctgatgaa

1078 gatgcacaca acatacagac ccacagagac agcttggatt tgctggacaa agcccgagca

1138 aagaaagcca tgttgtctga acaaaatagg gcttctcctc tccccagtgg gctcctcacc

1198 ccgccacaga gcggtaagaa gcagagcagc gggccggaaa tggcgtga

Underlined bases: Antisense cyc E ribozyme target sense sequence
Bold bases: Ribozyme target cleavage sequence
Boxed bases: C-5 propyne modified antisense oligonucleotide target

Cell 66 (6), 1217-1228 (1991)

Figure 5

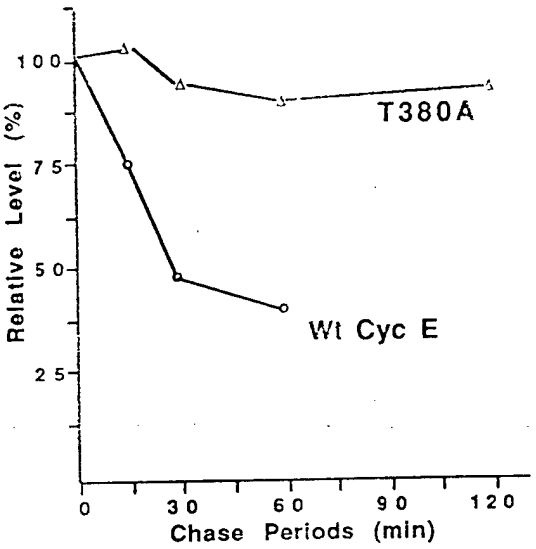

100
75
50
25
Relative Level (%)
T380A
Wt Cyc E
0
30
60
90
120
Chase Periods (min)

Figure 6

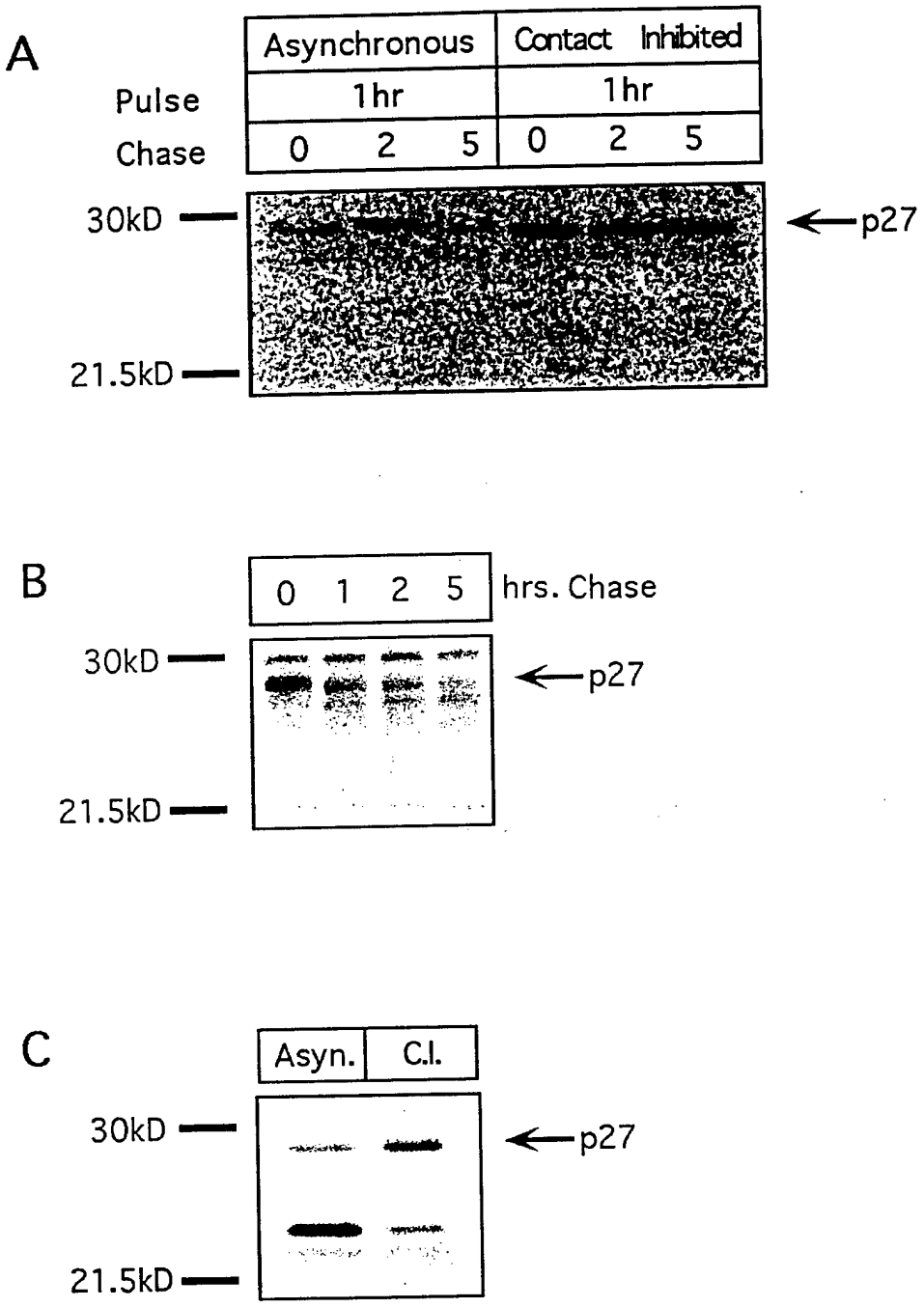

A
Asynchronous
Contact Inhibited
Pulse
1hr
1hr
Chase
0 2 5 0 2 5
30kD
21.5kD
p27
B
0 1 2 5 hrs. Chase
30kD
21.5kD
p27
C
Asyn.
C.I.
30kD
21.5kD
p27

Figure 7

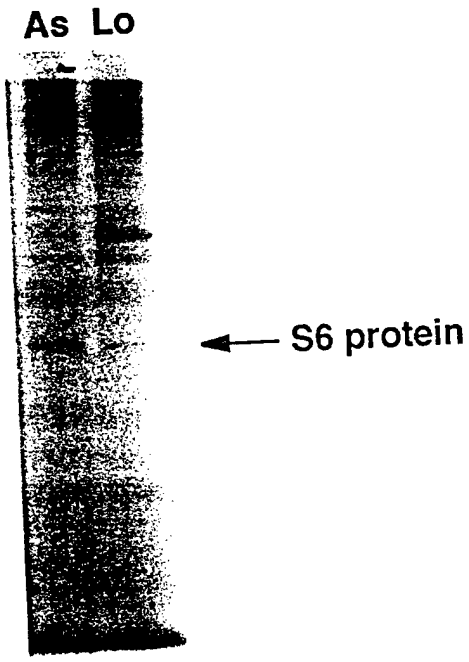
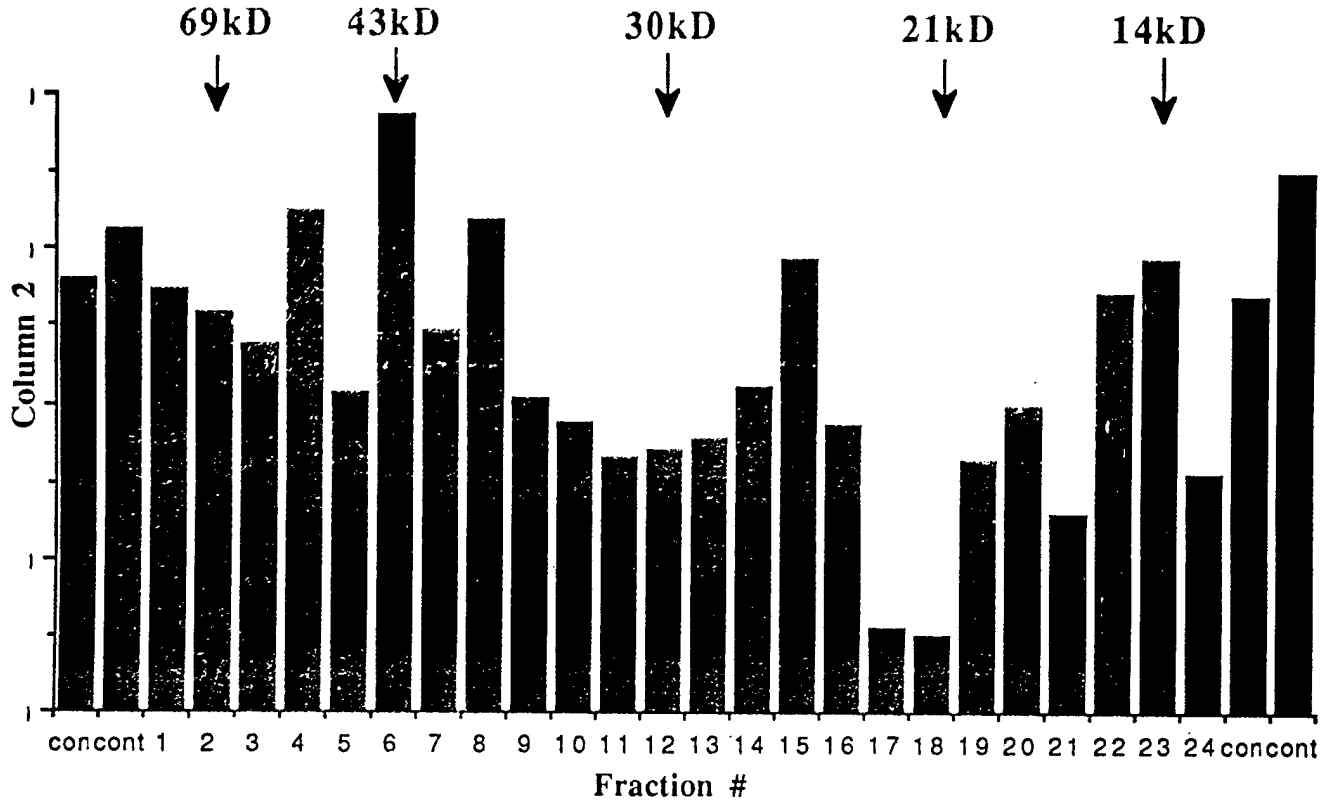


Figure 8

p28 5'UTR

Figure 9


69kD
43kD
30kD
21kD
14kD
Column 2
concont 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 concont
Fraction #

PERSONNEL

Steven I. Reed	20%
Meira Wolff	100%
Kwang-Ai Won	100%